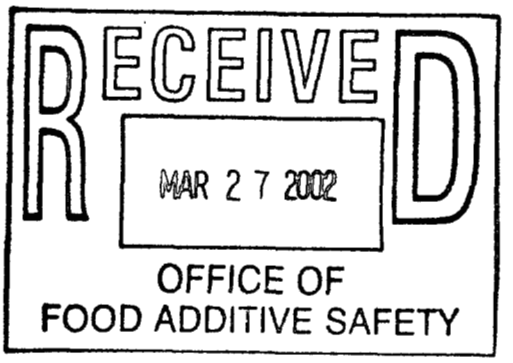
GR 

Original Submission

000001



novozymes®

March 25, 2002

Linda S. Kahl, Ph.D.
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' lipase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus and Fusarium oxysporum*. The lipase enzyme preparation is intended for use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks and whole eggs.

Please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Enclosures (3 binders)

000002

908654

March 22, 2002

novozymes®

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that lipase preparations produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus and Fusarium oxysporum* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Lipase enzyme preparation from *Aspergillus oryzae* expressing the gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus and Fusarium oxysporum.*

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The lipase is intended for use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks or whole eggs. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

25 March, 2002
Date

John Carroll
Director, Regulatory Affairs

000003

908654

novozymes®

A lipase preparation produced by *Aspergillus oryzae* expressing a gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus/Fusarium oxysporum*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA
Peter Hvass, Regulatory Affairs, Novozymes A/S, Denmark

TABLE OF CONTENTS

000005

908654

1. GENERAL INTRODUCTION

Lipopan® H and Lecitase® Ultra are Novozymes A/S trade names used for a lipase preparation produced by submerged fermentation of *Aspergillus oryzae* carrying a modified gene coding for a *Thermomyces lanuginosus/Fusarium oxysporum* hybrid lipase.

The enzyme preparation is to be used in the food industry (the baking and the oils and fats industries) as a processing aid. The trade name Lipopan H is used for the baking industry, whereas Lecitase Ultra is used for the oils and fats industry.

The enzyme is a lipase (EC 3.1.1.3), which specifically acts on the fatty acid in position 1 in both triglyceride substrates and phospholipids.

In the baking applications, both the activity towards triglyceride and phospholipid substrates are utilized to obtain improved dough properties and improved bread making quality in terms of larger volume and improved crumb structure.

The applications within the oils and fats industry are mainly modification of egg yolk or whole egg, hydrolysis of lecithin for altered emulsifying properties and vegetable oil de-gumming. In these applications, the activity towards the phospholipids (phosphatides, lecithins) will predominate due to the specific processing conditions.

The information provided in the following sections is the basis for our determination of general recognition of safety of a lipase enzyme preparation produced by *A. oryzae* expressing a gene encoding a modified/hybrid lipase from *T. lanuginosus/F. oxysporum*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[1,2]. The production organism for this Lipase, *A. oryzae*, is discussed in Sections 2 and 7. An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material[3-8]. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. Data showing this modified lipase to be substantially equivalent [5,7,8-11] to naturally occurring lipases is provided in Section 3. The safety studies performed and described in Section 7 show no evidence to indicate that any of the cloned DNA sequences and incorporated DNA encode or express a harmful or toxic substance.

It should be noted that in some reports, the enzyme product is designated "HL-1232" or batch "PPW 7023". HL-1232 is an internal name used during development. PPW 7023 is the internal code of the test batch used for safety testing.

000007

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *A. oryzae* strain, designated pCaHj559/BECh2#3, was constructed by plasmid transformation of the recipient strain, designated BECh2 (see Section 2.2). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms[12]. It also meets the criteria for a safe production microorganism as described by Pariza and Foster and several expert groups[3-8].

The lipase expression plasmid, pCaHj559 used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding a *T. Lanuginosus/F. oxysporum* hybrid lipase enzyme; an *A. nidulans* selectable marker gene, *amd*S (acetamidase)[13]; well-characterized noncoding regulatory sequences including the *A. niger* terminator[14], the *A. niger* NA2 promoter[15], and *A. nidulans* triose phospate isomerase gene[16]; a 1163 bp fragment from the *Escherichia coli* cloning vector pUC19[17], and the *Saccharomyces cerevisiae* URA3[18] promoter (Pura3), coding sequence (URA3) and terminator (Tura3).

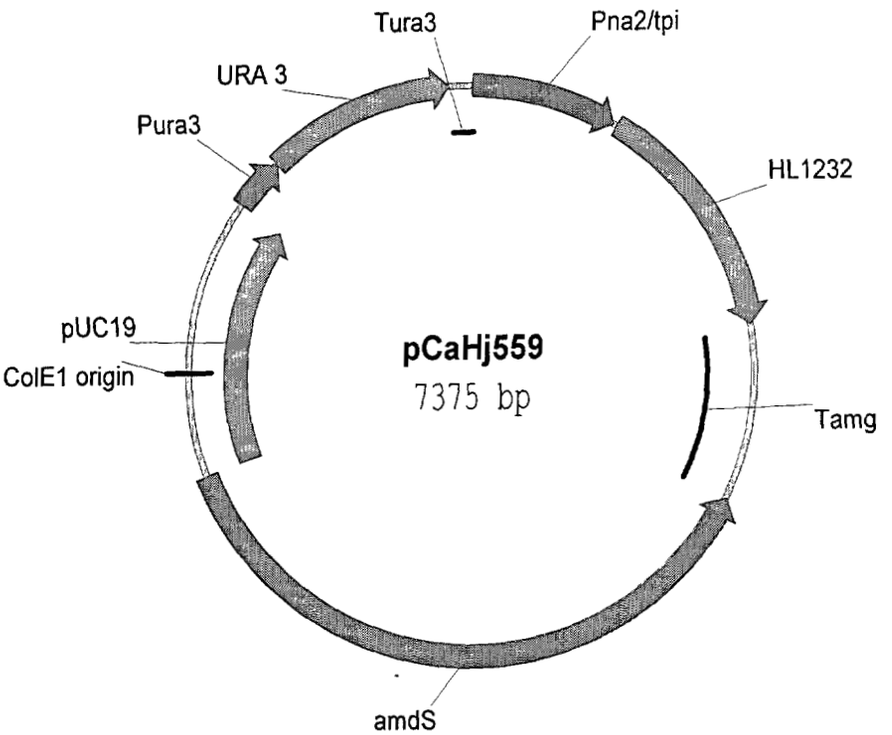
The *T. lanuginosus* lipase gene was modified by well-known techniques designed to introduce specific desired modifications in the coding sequence[19-20]. The identity and location of these changes were verified several times by DNA sequencing. The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and to ensure that no unintended sequences were incorporated in the production strain.

2.2 Recipient Organism

The recipient microorganism, designated BECh2, used in the construction of the lipase production strain is an amylase negative, alkaline protease (alp) negative, neutral metalloprotease I (NpI) negative, cyclopiazonic acid deficient, kojic acid deficient derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560). This classification of A1560 as *A. oryzae* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland[21].

2.3 Lipase Expression Plasmid

The7375 bp HL 1232 expression plasmid pCaHj559 consists of the following elements:



Plasmid map of pCaHj559

Position (bp)	Size (bp)	Element	Origin
1-616	616	Pna2/TPI	*A. niger* BO1
617-627	11	Linker	Synthetic
628-1644	1017	HL1232	*T. lanuginosus*, *F. oxysporum*
1645-1669	45	Linker	Synthetic
1670-2379	710	Tamg	*A. niger* BO1
2380-5104	2725	amdS	A. nidulans.
5105-6267	1163	pUC19	*E. coli.*
6268-6490	223	Pura3	*S. cerevisiae.*
6491-7291	801	URA3	*S. cerevisiae.*
7292-7375	84	Tura3	*S. cerevisiae.*

Pna2/tpi is the neutral amylase II promoter from *Aspergillus niger*. The 5' nontranslated part of this promoter has been replaced with the 5' nontranslated part of the *Aspergillus nidulans* triose phosphate isomerase (TPI) promoter (position 550-616).

HL1232 is the hybrid lipase.

Tamg is the amyloglycosidase terminator gene of *Aspergillus niger*.

*amd*S is the complete acetamidase encoding gene(including promoter and terminator) from *Aspergillus nidulans*.

pUC19 is a fragment of the pUC19 vector including the origin of replication. The origin of replication initiation site (colE1 origin) is in position 5539.

Pura3, URA3 and Tura 3 are the *Saccharomyces cerevisiae* URA3 promoter, coding sequence and terminator respectively.

The full DNA sequence of the plasmid is provided in Appendix 1.

2.4 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism[3-8]. The transforming DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[12].

2.5 Antibiotic Resistance Gene

The DNA used for transforming the *A. oryzae* host strain does not contain any antibiotic resistance genes.

2.6 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC[3] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme identity

Key enzyme and protein chemical characteristics of the lipase are given below:

Classification	Lipase (generic name)
IUB nomenclature:	Triacylglycerol lipase
IUB No.:	3.1.1.3
CAS No.:	9001-62-1
EINECS No.:	232-619-9
Specificity:	1,3-position ester bonds in triglycerides; specificity towards fatty acid in position 1 in both triglycerides and phospholipids.
Molecular weight:	35 kDa
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

3.2 Amino Acid Sequence

The Lecitase Ultra/Lipopan H lipase enzyme (HL 1232) has a higher preference for long chain fatty acids in position 1 in the substrate. The starting point for HL 1232 was the *T. lanuginosus* lipase (TII) which is the subject of GRAS notice GRN No. 43. HL 1232 is a 339 amino acid fusion protein between the N-terminal part of TII and the C-terminal part of the *F. oxsporum* lipase (FoL) which is the subject of GRAS notice GRN No. 75. The sequence of TII was changed at three specific amino acid residues:

-Glycine in position 113 has been altered to alanine.
-Aspartic acid in position 118 has been altered to tryptophan.
-Glutamic acid in position 121 has been altered to lysine.

Except for these 3 point mutations, the amino acid residues 1–284 originate from the N-terminal part of the mature TII and the amino acid residues 285–339 originate from the C-terminal part of the mature FoI. The protein sequence of HL1232 and the parent lipase sequences are shown in Appendix 2.

000011

The lipase's specificity toward long chain fatty acids in position 1 is utilized in baking applications where the lower tendency to releasing short chain fatty acids from primary ester bonds in triglycerides and phospholipids decreases the risk of off-flavor generation especially in baking formulas that contain butterfat. In fats and oils applications this enzyme has increased activity towards phospholipids and higher efficacy when used for the modification of egg yolk or whole egg, hydrolysis of lecithin for altered emulsifying properties and vegetable oil degumming.

3.3 Comparison to other lipases

Lipases are classified into four main families according to their structural homology. The general structure of known lipases is characterized by four major structural, functional or sequence homologies: 1) the consensus sequence Glycine-X-Serine-X-Glycine around the active-site serine; 2) the strand-helix motif around the active serine residue; 3) a buried active site covered by a lid or lids; 4) the active site catalytic residues found in a triad in the order Serine...Asparagine/Glutamine...Histidine, lying 50 residues from one another in the sequence[22].

T. lanuginosus lipase (Tll) and *Rhizomucor miehei* lipase (Rml) both belong to the *R. miehei* family[23]. The amino acid sequences of Rml and Tll lipases have been compared[24,25]. They are composed of the same number of amino acid residues, and there are substantial structural similarities. The 3D structure of the Tll enzyme is essentially identical to that of the Rml.

F. oxysporum lipase (Fol) is structurally homologous to Tll and Rml. The amino acid sequences of the Fol, Tll and Rml lipases have been compared[26]. Fol is 59% homologous to Tll and 49% homologous to Rml. Generally a sequence homology below 50% would indicate possible differences in the structural organisation, and above 50% would indicate a possible relationship in structure. By comparing the Tll, FoL and HL 1232 lipase sequences (see Appendix 1) it can be seen that the pentapeptide consensus sequence and the active site catalytic residues are the same in all three lipases. There are also conserved residues in the lid and hinge regions. The structural relation is also proved by 3D structural elucidation, showing that Fol has the same overall structure as Tll and Rml and thus is considered to be in the same lipase family.

The HL1232 enzyme is substantially equivalent to the *T.lanuginosus* and the *F. oxysporum* lipases as evaluated by sequence homology assessment. This is confirmed by enzymatic activity determination and application tests showing similar catalytic properties and functional effects in lipid modification.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the Lecitase Ultra and Lipopan H which follows standard industry practices[27-29]. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the lipase enzyme concentrate are standard ingredients used in the enzyme industry[27-29]. The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the Lipase product is less than 1%.

4.2 Fermentation Process

The lipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

4.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae,* described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by

microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy
2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

4.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Preservation and Stabilization – sodium chloride addition
6. Final concentration – evaporation if enzyme concentration is too low to reach target yield

4.3.2 Formulation and Standardization Processes

Lipase formula for fats and oils applications (Lecitase Ultra)

The stabilized concentrate is blended with water, sorbitol and potassium sorbate. The product is standardized according to the product specification.

Lipase formula for baking applications (Lipopan H)

The liquid concentrate is mixed with granulation aids (stabilizers/binders) such as dextrin and spray dried by means of atomization into a fluidized spray dryer. The powder from the primary drying zone is directed into an integrated fluid bed for

agglomeration and further drying. The product is discharged continuously after sieving. The product is standardized to the declared enzyme activity by addition of wheat flour.

4.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

5. COMPOSITION AND SPECIFICATIONS

The lipase enzyme preparation is presently available in formulas for use in fats and oils applications and baking applications.

5.1 Quantitative Composition

Lipase for fats and oils applications (Lecitase Ultra) has the following typical composition:

Enzyme solids (TOS)	approx.	5 %
Sorbitol	approx.	50 %
Sodium chloride	approx.	2 %
Water	approx.	43 %
Potassium sorbate	approx.	0.2 %

Lipase for baking applications (Lipopan H) has the following typical composition:

Enzyme solids (TOS)	approx.	2 %
Wheat solids	approx.	76 %
Sodium chloride	approx.	12 %
Dextrin	approx.	3 %
Water	approx.	7 %

5.2 Specifications

The Lipase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 4th edition, 1996[30]. In addition, the Lipase also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[31].

The following Novozymes' specifications have been established for the Lipase:

Enzyme activity, KLU/g*	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 5×10^4
Total coliforms/g	not more than 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella/25 g	negative by test
Antibiotic activity	negative by test
Production organism	negative by test
Mycotoxins	negative by test

Heavy metals, lead, arsenic, antibiotic activity, and mycotoxins are analyzed at regular intervals.

The heavy metals and lead specifications meet FCC and exceed JECFA requirements. The arsenic, total viable count and *E.coli* specifications meet JECFA requirements and are not included in FCC. The total coliforms and *Salmonella* specifications meet both FCC and JECFA. The antibiotic activity and mycotoxins specifications meet JECFA and are not included in FCC (although FCC mentions mycotoxins but has not established tolerances). The production microorganism specification is a Novozymes' specification and is not mentioned in FCC or JECFA.

*The activity of this enzyme is measured in KLU/g. The LU method is based on the speed at which the enzyme hydrolyses tributyrin at pH 7.0. The butyric acid formed is titrated with sodium hydroxide and the consumption of the latter recorded as a function of time. 1 LU is the amount of enzyme, which releases 1 µmol butyric acid per minute under the given standard conditions. 1 KLU = 1000 LU.

6. APPLICATION

6.1 Mode of Action

The enzyme is a lipase (EC 3.1.1.3). This product hydrolyzes ester bonds of triacylglycerol to release free fatty acids. It also catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids and forms 2-acyl-1-lysophospholipid and a free fatty acid.

Lipopan H will be used for the baking applications, whereas Lecitase Ultra will be used in the fats and oils industry for modification of egg yolk or whole egg, hydrolysis of

lecithin for altered emulsifying properties and de-gumming of vegetable oil. In the baking applications, both the activity towards triglyceride and phospholipid substrates is utilized, while in the fats and oils applications, the activity towards the phospholipids (phosphatides, lecithins) will predominate due to specific processing conditions.

In the baking application, the Lipase is used to obtain improved dough properties and improved bread making quality in terms of larger volume and improved crumb structure.

In the "degumming" of oil, phospholipids (phosphatides, lecithins) are removed from oil during the oil purification process in order to improve the taste and quality of the oil. Removal of phospholipids can also improve the storage stability of the oil and facilitate downstream processing. The Lipase is applied in an aqueous phase in oil. This aqueous phase is typically relatively low e.g. around 5 % in oil. Phospholipids hydrolyzed with the Lipase migrate into the aqueous phase, and can be removed as a water based sludge.

The Lipase can also be used to hydrolyze lecithin. Lysolecithin, 2-acyl-1-lysophophospholipid, as a product of enzymatic hydrolysis of lecithin, has excellent emulsifying properties. The lipase can also hydrolyze the lecithin in egg yolks improving the emulsifying properties of the egg yolks.

The product sheet for Lecitase Ultra is included in Appendix 3.

6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect. For the baking applications, the recommended dosage of Lipopan H is up to 1.5 KLU per kg flour, corresponding to 0.5 g of Lipopan H per kg flour.

In the oils and fats applications, the optimum dosage is dependent on the specific substrate, reactor configuration and desired conversion etc. However, the recommended dosage of Lecitase Ultra would be:

a) up to 40 KLU per kg of egg yolk or whole egg, corresponding to 1 kg of Lecitase Ultra to produce 250 kg of modified egg yolk / whole egg
b) up to 25 KLU per kg of crude lecithin for lecithin hydrolysis, corresponding to 1 kg of Lecitase Ultra to produce 400 kg of modified lecithin
c) up to 0.5 KLU per kg of triglycerides for de-gumming, corresponding to 1 kg of Lecitase Ultra to produce 20 ton of de-gummed oil

6.3 Enzyme Residues in the Final Food

Baking

Lipopan H is added to the flour or the liquid and is active during the dough preparation and the leavening of the unbaked bread. During the baking process the high temperatures in the oven cause an inactivation of the enzyme activity.

Modification of egg yolk / whole egg and lecithin hydrolysis
After the reaction, the enzyme is inactivated by heat treatment.

De-gumming
In the de-gumming application, Lecitase Ultra is applied in a water phase to the oil. The enzyme is soluble in water and insoluble in organic solvents, and will not migrate into the oil phase. After the reaction, the water phase, containing Lecitase Ultra and lysophospholipids, is removed. Furthermore the oil will be subjected to oil refining methods, which would remove any potential residues.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[2]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume[3]. Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA[32].

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes[33]. *A. oryzae* is accepted as a constituent of foods[32]. *A. oryzae* has been used to produce soy sauce in the United States since before 1958[3,33]. Therefore, *A. oryzae* meets the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS[3]. A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973[34] (They are also the subject of GRN no. 90). Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS by Novozymes and other companies since that time. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS[2,3,32].

An evaluation of the genetically modified production microorganism for the Lipase, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 and Pariza and Johnson, 2001 demonstrates the safety of this genetically modified production microorganism

strain. The components of this evaluation: the identity of the host strain, a description of the plasmid used, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2 and 3.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the lipase enzyme preparation derived from the genetically modified *A.oryzae* is considered safe[3,9]. To confirm the safety of the enzyme, safety studies were performed on the enzyme preparation and are described in Section 7.5.

7.2 Safety of the donor organisms

The lipase enzyme from the genetically modified *A. oryzae* is a hybrid lipase from *T. lanuginosus* and *F. oxysporum. T. lanuginosus* is an ubiquitous, thermophilic fungus that is not described as pathogenic or as a known toxin producer[35]. *F. oxysporum* belongs to the section Elegans of the genus *Fusarium* within the class of imperfect fungi Hyphomycetes. In general, these fungi are not regarded as primary human pathogens. They are adapted to a living as saprophytes and many of them as plant parasites. Fungal spores or hyphal fragments, which are deposited on the skin or enter the body via one of its orifices, are generally incapable of further growth and development. Additional information on *F. oxysporum* is available in GRN No. 75.

However, the pathogenic and toxigenic potential of the particular strains used as donor for the lipase gene should not be of particular importance to the safety of our lipase enzyme preparation[3]. Pariza and Johnson[1]confirm that the safety of the production strain is the primary concern in evaluating enzyme safety. Only well characterized DNA fragments, limited solely to the lipase coding sequence from the donor strains, are used in the construction of the *A. oryzae* production strain. The introduced DNA does not code for any known harmful or toxic substances. The full DNA sequence of the lipase expression plasmid is given in Appendix 1.

7.3 Safety of the Lipase Enzyme

Enzyme proteins themselves do not generally raise safety concerns[2,36,37]. As indicated in section 3, the Lipase is a triacylglycerol lipase, IUB EC 3.1.1.3, which hydrolyzes the primary ester bonds in triglycerides. Most of the lipases commonly used in food processing belong to this group[36]. Like other known lipases[38], this lipase also catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids and forms 2-acyl-1-lysophospholipid and free fatty acid.

7.3.1 Lipases

Microbial lipases have been reported to be used in food production since 1952[39,40]. Animal lipase is affirmed as GRAS (21 CFR 184.1415) based on its common use in food prior to 1958. Esterase-lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive (21 CFR 173.140). Lipase enzyme from *Rhizopus niveus* is affirmed as GRAS based on scientific procedures (21 CFR 184.1420). Also, Novo Nordisk filed a petition in 1989 proposing to affirm that insoluble esterase-lipase enzyme preparation derived from *Mucor miehei* which has been fixed by immobilization with a substance that is generally recognized as safe or an approved food additive is GRAS for use as a direct human food ingredient (54 FR 9565). Esterase lipase enzyme preparations from *Mucor miehei* have been marketed by Novozymes as GRAS since that time. A lipase preparation produced by *A. oryzae* expressing the gene encoding a lipase from *T. lanuginosus* is the subject of GRAS notification GRN #43. Also, a lipase preparation produced by *A. oryzae* expressing the gene encoding a lipase from *F. oxysporum* is the subject of GRAS notification GRN #75.

7.3.2 Substantial Equivalence

Several expert groups, as well as FDA and FDA scientists have discussed the concept of substantial equivalence relative to food safety assessment[5-11,41]. Essentially all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food[10,42,43]. In particular, differences in glycosylation between enzyme proteins was considered. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to changes in the enzyme amino acid sequence by natural selection, chemical modification, or site-directed mutagenesis would not raise safety concerns[9,11].

The Lecitase Ultra/Lipopan H lipase enzyme from the genetically modified *A. oryzae* is substantially equivalent to the *T. lanuginosus* and *F. oxysporum* lipases. The Lecitase Ultra/Lipopan H lipase is functionally equivalent and within the range of natural variation of lipases (See section 3).

As discussed in Section 3.2, the HL1232 enzyme is a lipase that is substantially equivalent to the *Thermomyces lanuginosus* and the *Fusarium oxysporum* lipases as evaluated by sequence homology assessment. Each of these lipases have been expressed in *Aspergillus oryzae* production strains, and exist as commercial enzyme products from Novozymes A/S that are widely accepted as safe for use within the food

industry. The HL 1232 lipase has a slightly altered specificity towards long chain fatty acids compared to short chain fatty acids in position 1 in the substrate. This is confirmed by enzymatic activity determination and application tests showing similar catalytic properties and functional effects in lipid modification.

7.4 Safety of the Manufacturing Process

The Lipase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 4, the lipase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes[27-29].

7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the Lipase.

7.5.1 Description of Test Material

The safety studies described below were conducted on a liquid lipase enzyme concentrate that was prepared according to the description given in Section 4 except that stabilization and standardization were omitted.

7.5.2 Studies

The following studies were performed:

-13 weeks Subchronic Oral Toxicity in rats
-Test for mutagenic activity (Ames test)
-Human lymphocyte cytogenetic assay

A summary of the safety studies performed on the lipase is included in Appendix 4.

7.6 Estimates of Human Consumption and Safety Margin

As stated in section 6.3 the enzyme activity is largely heat inactivated and/or removed during the baking process, the process used to obtain egg yolk / whole egg modification, the lecithin hydrolysis process or the de-gumming process of vegetable oil.

However, in order to illustrate a "worst case" situation the following calculations are made assuming that all enzyme activity is retained in the baking product, the modified egg yolk / whole egg, the hydrolyzed lecithin and the vegetable oil, respectively.

Lipopan H has an activity of 3 KLU/g and an approximate content of 2% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components). Lecitase Ultra has an activity of 10 KLU/g and an approximate content of 5 % TOS.

Baking

The average human intake of bread is estimated using well-established statistics from various countries.

United Kingdom

The Ministry of Agriculture, Fisheries and Food: 1987 Annual Report of the National Food Survey Committee, Household Food Consumption and Expenditure: Consumption of bread, cakes and biscuits per person per day is 158 g.

Denmark

"Levnedsmiddelstyrelsen": Development of Food Consumption in Denmark, 1955-1990, Description of the Danish Diet based on food statistics and nutrition calculated data: Consumption of bread, flutes, pita-bread, cakes, and rye bread per person per day is 123 g.

USA

Industrial Outlook 1992 (Food Beverages): Consumption of bread and related products per person per day is 109 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is made assuming that all TOS remains in the baking product. Lipopan H contains 2 % TOS.
b) It is assumed that all baking products are produced using Lipopan H as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of Lipopan H is 1.5 KLU per kg flour, corresponding to 0.5 g of Lipopan H per kg flour as described in Section 6.2. Lipopan H contains 2 % TOS. Using a standard recipe, 100 kg flour results in 140 kg bread, giving a theoretical content of 7.1 mg TOS/kg bread.

Based on the highest average daily intake of baking products (158 g), the daily intake per person of Lipopan H corresponds to 7.1 x 0.158 = 1.13 mg TOS per day.

For an average person weighing 60 kg this corresponds to 1.9×10^{-5} g TOS/ kg body weight per day.

Egg yolk / whole egg modification

The modified egg yolk has a superior emulsifying effect. It is used for e.g. mayonnaise.

In accordance with FDA 21 CFR §101.12 Reference amount customarily consumed per eating occasions, consumption of mayonnaise per person per eating occasions is 15 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is made assuming that all TOS remains in the modified egg yolk. Lecitase Ultra contains 5 % TOS.
b) It is assumed that mayonnaise is consumed at three eating occasions a day. It corresponds to 45 g mayonnaise daily per person. Mayonnaise contains typically 5 % yolk, corresponding to 2.25 g egg yolk per person per day.
c) It is further assumed that all mayonnaise is produced using modified egg yolk, which is processed with Lecitase Ultra at the highest recommended dosage level.

The maximum recommended dosage of Lecitase Ultra is 40 KLU/kg egg yolk, corresponding to 1 kg for 250 kg of modified egg yolk as described in Section 6.2. Lecitase Ultra contains 5 % TOS. Therefore, the modified egg yolk would contain 200 mg TOS/kg egg yolk.

Based on the daily intake of mayonnaise (corresponding to 2.25 g egg yolk per day), the daily intake per person of Lecitase Ultra corresponds to 200 x 0.00225 = 0.45 mg TOS per day.

For an average person weighing 60 kg this corresponds to 7.5×10^{-6} g TOS/ kg body weight per day.

Lecithin hydrolysis

Lecithin is a food additive used widely for food[44]. According to Lehman[45], the average human intake of food per day is 1500 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is made assuming that all TOS remains in the hydrolyzed lecithin product. Lecitase Ultra contains 5 % TOS.
b) It is assumed that half of the human intake of food contains food additives. It corresponds to 750 g as the average human intake of the food containing food additives per day.
c) It is further assumed that all this 750 g of modified food per day contains 0.5 % lecithin, which is processed with Lecitase Ultra at the highest recommended dosage level, corresponding to 3.75 g lecithin per day.

The maximum recommended dosage of Lecitase Ultra is 25 KLU/kg crude lecithin, corresponding to 1 kg for 400 kg of hydrolyzed lecithin as described in Section 6.2. Lecitase Ultra contains 5 % TOS. Therefore, the hydrolyzed lecithin would contain 125 mg TOS/kg lecithin.

Based on the daily intake of food containing food additives (corresponding to 3.75 g lecithin per day), the daily intake per person of Lecitase Ultra corresponds to 125 x 0.00375 = 0.47 mg TOS per day.

For an average person weighing 60 kg this corresponds to 7.8×10^{-6} g TOS/ kg body weight per day.

De-gumming

The average human intake of oils and fats is estimated using well-established statistics from various countries.

United Kingdom

The Ministry of Agriculture, Fisheries and Food: 1987 Annual Report of the National Food Survey Committee, Household Food Consumption and Expenditure: Consumption of oil & fat excluding butter per person per day is 32 g.

Denmark

"Levnedsmiddelstyrelsen": Development of Food Consumption in Denmark, 1955-1990, Description of the Danish Diet based on food statistics and nutrition calculated data: Consumption of oil and fat per day is 144 g.

USA

The Economics Research Service, USDA: Consumption of vegetable oil per person per day is 69 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

000024

a) The calculation is made assuming that all TOS remains in the oil, although it would not be the case in reality. Lecitase Ultra contains 5 % TOS.
b) It is assumed that all oils and fats are produced using Lecitase Ultra as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of Lecitase Ultra is 0.5 KLU/kg triglycerides, corresponding to 1 kg for 20 ton de-gummed oil as described in Section 6.2. Lecitase Ultra contains 5 % TOS. Therefore, the triglycerides would contain 2.5 mg TOS/kg triglyceride.

Based on the highest average daily intake of oils and fats (144 g), the daily intake per person of Lecitase Ultra corresponds to 2.5 x 0.144 = 0.36 mg TOS per day.

For an average person weighing 60 kg this corresponds to 6.0×10^{-6} g TOS/ kg body weight per day.

Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption.

The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kg/day corresponding to 1.02 g TOS/kg/day.

The estimated human consumption is:

Baking:	1.9×10^{-5} g TOS/kg/day
Egg yolk modification:	7.5×10^{-6} g TOS/kg/day
Lecithin hydrolysis:	7.8×10^{-6} g TOS/kg/day
De-gumming:	6.0×10^{-6} g TOS/kg/day

The safety margin can thus be calculated to be:

Baking:	$1.02/1.9 \times 10^{-5} = 5.4 \times 10^{4}$
Egg yolk modification:	$1.02/7.5 \times 10^{-6} = 1.4 \times 10^{5}$
Lecithin hydrolysis:	$1.02/7.8 \times 10^{-6} = 1.3 \times 10^{5}$
De-gumming:	$1.02/6.0 \times 10^{-6} = 1.7 \times 10^{5}$

000025

7.7 Results and Conclusion

The results of the tests described in section 7.4.2 show that the Lipase enzyme preparation does not exhibit any mutagenic activity, clastogenic activity, or toxic effect under the conditions of each specific test. On the basis of the evaluation contained in Sections 7.1-7.5, a review of the published literature, the history of use of *A. oryzae,* and the limited and well defined nature of the genetic modifications, the Lipase enzyme preparation can be safely manufactured and used as a processing aid in the fats and oils and baking industries as well as in other food or non-food applications.

8. LIST OF APPENDICES

1. Full DNA sequence of the plasmid pCaHj 559
2. The amino acid sequence for the enzyme protein in Lipopan H/Lecitase Ultra, compared with the protein sequence of the *Thermomyces lanuginosus* lipase and the *Fusarium oxysporum* lipase.
3. Product Sheet for Lecitase Ultra
4. Summary of Toxicity Data, File 2002-03562-01

9. LIST OF REFERENCES

1. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.

2. Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46:5:453-468, 1983.

3. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regulatory Toxicology and Pharmacology 12:S1-S196. 1990.

4. EU Scientific Committee for Food. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series, 1991.

5. Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.

6. Berkowitz, D. and Maryanski, J.. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3-12, 1989.

7. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy. 1996

8. Jonas, D.A., Antignac, E., Antoine, J.M., Classen, H.G., Huggett, A., Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R., and de Vogel, P.. The Safety Assessment of Novel Foods, Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chemical Toxicology 34:931-940. 1996.

9. Kessler, D.A., Taylor, M.R., Maryanski, J.H., Flamm, E.L., and Kahl, L.S.. The Safety of Foods Developed by Biotechnology. Science 256:1747-1749, 1832. 1992.

10. Food and Drug Administration. Enzyme preparations from animal and plant sources; Affirmation of GRAS status as direct food ingredients. Fed. Regist. 60:32904-32912, 1995.

11. Food and Drug Administration. Substances generally recognized as safe. Proposed Rule. Fed. Regist. 62:18938-18964, 1997.

12. Organisation for Economic Co-operation and Development ,Safety Considerations for Biotechnology, 1992.

13. Hynes, M.J., Corrick, C.M. and King, J.A.. Isolation of Genomic Clones Containing the amdS Gene of Aspergillus nidulans and Their Use in the Analysis of Structural and Regulatory Mutations, Mol. Cell Biol. 2:1430-1439, 1983.

14. Boel, E., Hansen, M.T., Hjort, I., Hoegh, I., and Fiil, N.P.. Two different types of intervening sequences in the glucoamylase gene from Aspergillus niger, EMBO Journal, 3:1581-1585, 1984.

15. Novo Industri A/S. WO 89/ 01969 Patent. Process for the Production of Protein Products in *Aspergillus* and Promoters for Use in *Aspergillus.* March 9, 1989.

16. McKnight, G.L., O'Hara, P.J., and Parker, M.L.. Nucleotide Sequence of the Triosephosphate Isomerase Gene from *Aspergillus nidulans*: Implications for a Differential Loss of Introns. Cell 46: 143-147, 1986.

17. Yanisch-Perron, C., Vieira, J. and Messing, J.. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, Gene, 33:103-119,1985.

18. Invitrogen corporation, Carlsbad, CA, USA: http://www.invitrogen.com/Content/Tech-Online/molecular_biology/manuals_pps/pyes2_man.pdf

19. Higuchi R., Krummel, B., and Saiki, R.K.. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Research 16:7351-7367, 1988.

20. Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61-68, 1989.

21. Centraalbureau voor Schimmelcultures, Identification Report on Strain A1560, Baarn, Holland. 1988.

22. Svendsen, A.. "Sequence comparisons within the lipase family," in Lipases their structure, biochemistry and application, edited by Woolley, P. and Petersen, S.B., Cambridge University Press, Cambridge, pp 1-21, 1994.

23. Schmidt-Dannert, C., Recombinant Microbial Lipases for Biotechnological Applications. Bioorganic & Medicinal Chemistry, 7:2123-2130, 1999.

24. Lawson, D.M., Brzozowski, A.M., Dodson, G.G., Hubbard, R.E., Huge-Jensen, B., Boel, E., Derewenda, Z.S., "The three-dimensional structures of two lipases from *filamentous fungi*", Lipases their structure, biochemistry, and application, edited by Woolley, P., and Petersen, S.B., Cambridge University Press, Cambridge, pp. 77-94, 1994.

25. Derewenda, U., Swenson, L., Wei, Y., Green, R., Kobos, P.M., Joerger, R., Haas, M.J., and Derewenda, Z.S. Conformational lability of lipases observed in the absence of an oil-water interface: crystallographic studies of enzymes from the fungi *Humicola lanuginose* and *Rhizopus delemar.* Journal of Lipid Research 35:524-534, 1994.

26. Novo Nordisk A/S. WO 00/32758 Patent. Lipolytic Enzyme Variants. June 8, 2000.

27. Enzyme Applications in Encyclopedia of Chemical Technology, 4th ed., Ed. Kroschwitz, J.I., Volume 9, pp. 567-620, 1994.

28. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K.. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Vol. I, Eds. Peppler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.

29. Aunstrup, K.. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.

30. National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemicals Codex, Food Chemicals Codex, 4th Edition, National Academy Press, Washington, D.C., p. 133, 1996.

31. JECFA (Joint FAO/WHO Expert Committee on Food Additives), General Specifications for Enzyme Preparations Used in Food Processing, Compendium of Food Additive Specifications, Vol. 1, Annex 1, FAO, 1992 as supplemented in Appendix B to Annex 1, FAO FNP (Food and Nutrition Paper) 52, Add.6, Geneva, 1998.

32. Toxicological Evaluation of Certain Food Additives, 31st Meeting of the Joint FAO/WHO Expert Committee on Food Additives, Geneva, Switzerland, February 16-25, 1987.

33. Barbesgaard, Peder, Heldt-Hansen, Hans Peter and Diderichsen, Børge.. On the Safety of *Aspergillus oryzae:* a Review, , Appl. Microbiol. Biotechnol., 36:569-572, 1992.

34. Food and Drug Administration. Notice of Filing of Petition for Affirmation of GRAS Status. Fed. Regist. 38:1547, 1973.

35. Cooney, D.G. and Emerson, R., "Thermophilic Fungi", W.H. Freeman and Company, pp 82-87, 1964.

36. Food and Drug Administration. Lipase Enzyme Preparation From *Rhizopus niveus*: Affirmation of GRAS status as a Direct Food Ingredient. Fed. Regist. 63:24416-24419, 1998.

37. Food and Drug Administration. Statement of Policy: Foods Derived From New Plant Varieties. Fed. Regist. 57:22984-23005, 1992.

38. Simons, J-M, F. A., van Kampen M. D., Riel, S., Götz, F., Egmond, M. R., and Verheil, H.. Cloning, purification and characterization of the lipase from *Staphylococcus epidermidis*. Eur. J. Biochem. 253:675-683, 1998.

39. Beckhorn, E.J., Labbe, M.D., and Underkofler, L.A.. Production and Use of Microbial Enzymes for Food Processing. J. Agr. Food Chemistry 13:30-34, 1965.

40. Rogers, R.W.. Monograph on Microbial Enzymes, Prepared for Food and Drug Administration, Washington, DC, Bureau of Foods, Informatics, Inc., Rockville, MD. pp 1-16, 1977.

41. Maryanski, J.H.. Center for Food Safety and Applied Nutrition, US Food and Drug Administration. *FDA's Policy for Foods Developed by Biotecnology,* In Genetically Modified Foods: Safety Issues, Eds. Engel, Takeoka, and Teranishi, American Chemical Society, Symp. Series No. 605, Ch. 2, pp. 12-22, 1995.

42. Food and Drug Administration. Microbially derived chymosin enzyme preparations: Affirmation of the GRAS status. Fed. Regist. 55:10932-10935, 1990. Fed. Regist. 57:6476-6479, 1992. Fed. Regist. 58:27197-27202, 1993.

43. Food and Drug Administration. Secondary Direct Food Additives Permitted in Food For Human Consumption: Milk-Clotting Enzymes. Fed. Regist. 62:59281-59284, 1997.

44. Lecithins: Sources, Manufacturing and Uses. Ed. Bernard F. Szuhaj, The American Oil Chemists' Society, Illinois, 1989.

45. Lehman, A. J.. Association of Food and Drug Officials Quarterly Bulletin, 18:66, 1954.

000031

[redacted]

Appendix 1

000031.001

Appendix 1 --

Complete DNA sequence of pCaHj 559.

```
                                Pna2/tpi
1     AATTCATGGTGTTTTGATCATTTTAAATTTTTATATGGCGGGTGGTGGGCAACTCGCTTGCGCGGGCAAC
                                Pna2/tpi
71    TCGCTTACCGATTACGTTAGGGCTGATATTTACGTAAAAATCGTCAAGGGATGCAAGACCAAAGTACTAA
                                Pna2/tpi
141   AACCCCGGAGTCAACAGCATCCAAGCCCAAGTCCTTCACGGAGAAACCCCAGCGTCCACATCACGAGCGA
                                Pna2/tpi
211   AGGACCACCTCTAGGCATCGGACGCACCATCCAATTAGAAGCAGCAAAGCGAAACAGCCCAAGAAAAAGG
                                Pna2/tpi
281   TCGGCCCGTCGGCCTTTTCTGCAACGCTGATCACGGGCAGCGATCCAACCAACACCCTCCAGAGTGACTA
                                Pna2/tpi
351   GGGGCGGAAATTTATCGGGATTAATTTCCACTCAACCACAAATCACAGTCGTCCCCGGTATTGTCCTGCA
                                Pna2/tpi
421   GAATGCAATTTAAACTCTTCTGCGAATCGCTTGGATTCCCCGCCCCTGGCCGTAGAGCTTAAAGTATGTC
                                Pna2/tpi
491   CCTTGTCGATGCGATGTATCACAACATATAAATACTGGCAAGGGATGCCATGCTTGGAGTTTCCAACTCA
                         Pna2/tpi                                    HL1232
561   ATTTACCTCTATCCACACTTCTCTTCCTTCCTCAATCCTCTATATACACAACTGGGGATCCTTCACCATG
                                HL1232
631   AGGAGCTCCCTTGTGCTGTTCTTTGTCTCTGCGTGGACGGCCTTGGCCAGTCCTATACGTAGAGAGGTCT
                                HL1232
701   CGCAGGATCTGTTTAACCAGTTCAATCTCTTTGCACAGTATTCAGCTGCCGCATACTGCGGAAAAAACAA
                                HL1232
771   TGATGCCCCAGCAGGTACAAACATTACGTGCACGGGAAATGCCTGCCCCGAGGTAGAGAAGGCGGATGCA
                                HL1232
841   ACGTTTCTCTACTCGTTTGAAGACTCTGGAGTGGGCGATGTCACCGGCTTCCTTGCTCTCGACAACACGA
                                HL1232
911   ACAAATTGATCGTCCTCTCTTTCCGTGGCTCTCGTTCCATAGAGAACTGGATCGCGAATCTTAACTTCTG
                                HL1232
981   GTTGAAAAAAATAAATGACATTTGCTCCGGCTGCAGGGGACATGACGGCTTCACTTCGTCCTGGAGGTCT
                                HL1232
1051  GTAGCCGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGGAGCATCCCGACTATCGCGTGGTGTTTA
                                HL1232
1121  CCGGACATAGCTTGGGTGGTGCATTGGCAACTGTTGCCGGAGCAGACCTGCGTGGAAATGGGTATGATAT
                                HL1232
1191  CGACGTGTTTTCATATGGCGCCCCCCGAGTCGGAAACAGGGCTTTTGCAGAATTCCTGACCGTACAGACC
                                HL1232
1261  GGCGGAACACTCTACCGCATTACCCACACCAATGATATTGTCCCTCGACTCCCGCCGCGCGAATTCGGTT
                                HL1232
1331  ACAGCCATTCTAGCCCAGAGTACTGGATCAAATCTGGAACCCTTGTCCCCGTCACCCGAAACGATATCGT
                                HL1232
1401  GAAGATAGAAGGCATCGATGCCACCGGCGGCAATAACCAGCCTAACATTCCGGATATCCCTGCGCACCTG
                                HL1232
1471  TGGTACTTCCAGGCGACTGACGCCTGTAACGCTGGTGGCTTCTCTTGGCGACGATACAGAAGCGCCGAGA
                                HL1232
1541  GCGTCGACAAGAGGGCCACCATGACTGATGCCGAGCTTGAGAAGAAGCTGAACTCTTATGTCCAGATGGA
                  HL1232                                               Tamg
1611  TAAGGAGTATGTGAAGAATAACCAGGCCCGCTCTTAACGAGGGTATGAGGTTTGATGGTCTAGAGCTCGA
```

000032

[redacted]

Tamg

1681 CCGCGACGGTGACTGACACCTGGCGGTAGACAATCAATCCATTTCGCTATAGTTAAAGGATGGGGATGAG

Tamg

1751 GGCAATTGGTTATATGATCATGTATGTAGTGGGTGTGCATAATAGTAGTGAAATGGAAGCCAAGTCATGT

Tamg

1821 GATTGTAATCGACCGACGGAATTGAGGATATCCGGAAATACAGACACCGTGAAAGCCATGGTCTTTCCTT

Tamg

1891 CGTGTAGAAGACCAGACAGACAGTCCCTGATTTACCCTGCACAAAGCACTAGAAAATTAGCATTCCATCC

Tamg

1961 TTCTCTGCTTGCTCTGCTGATATCACTGTCATTCAATGCATAGCCATGAGCTCATCTTAGATCCAAGCAC

Tamg

2031 GTAATTCCATAGCCGAGGTCCACAGTGGAGCAGCAACATTCCCCATCATTGCTTTCCCCAGGGGCCTCCC

Tamg

2101 AACGACTAAATCAAGAGTATATCTCTACCGTCCAATAGATCGTCTTCGCTTCAAAATCTTTGACAATTCC

Tamg

2171 AAGAGGGTCCCCATCCATCAAACCCAGTTCAATAATAGCCGAGATGCATGGTGGAGTCAATTAGGCAGTA

Tamg

2241 TTGCTGGAATGTCGGGGCCAGTTGGCCGGGTGGTCATTGGCCGCCTGTGATGCCATCTGCCACTAAATCC

Tamg

2311 GATCATTGATCCACCGCCCACGAGGCGCGTCTTTGCTTTTTGCGCGGCGTCCAGGTTCAACTCTCTCCTC

amdS

2381 TAGACTGGAAACGCAACCCTGAAGGGATTCTTCCTTTGAGAGATGGAAGCGTGTCATATCTCTTCGGTTC

amdS

2451 TACGGCAGGTTTTTTTCTGCTCTTTCGTAGCATGGCATGGTCACTTCAGCGCTTATTTACAGTTGCTGGT

amdS

2521 ATTGATTTCTTGTGCAAATTGCTATCTGACACTTATTAGCTATGGAGTCACCACATTTCCCAGCAACTTC

amdS

2591 CCCACTTCCTCTGCAATCGCCAACGTCCTCTCTTCACTGAGTCTCCGTCCGATAACCTGCACTGCAACCG

amdS

2661 GTGCCCCATGGTACGCCTCCGGATCATACTCTTCCTGCACGAGGGCATCAAGCTCACTAACCGCCTTGAA

amdS

2731 ACTCTCATTCTTCTTATCGATGTTCTTATCCGCAAAGGTAACCGGAACAACCACGCTCGTGAAATCCAGC

amdS

2801 AGGTTGATCACAGAGGCATACCCATAGTACCGGAACTGGTCATGCCGTACCGCAGCGGTAGGCGTAATCG

amdS

2871 GCGCGATGATGGCGTCCAGTTCCTTCCCGGCCTTTTCTTCAGCCTCCCGCCATTTCTCAAGGTACTCCAT

amdS

2941 CTGGTAATTCCACTTCTGGAGATGCGTGTCCCAGAGCTCGTTCATGTTAACAGCTTTGATGTTCGGGTTC

amdS

3011 AGTAGGTCTTTGATATTTGGAATCGCCGGCTCGCCGGATGCACTGATATCGCGCATTACGTCGGCGCTGC

amdS

3081 CGTCAGCCGCGTAGATATGGGAGATGAGATCGTGGCCGAAATCGTGCTTGTATGGCGTCCACGGGGTCAC

amdS

3151 GGTGTGACCGGCTTTGGCGAGTGCGGCGACGGTGGTTTCCACGCCGCGCAGGATAGGAGGGTGTGGAAGG

amdS

3221 ACATTGCCGTCGAAGTTGTAGTAGCCGATATTGAGCCCGCCGTTCTTGATCTTGGAGGCAATAATGTCCG

amdS

3291 ACTCGGACTGGCGCCAGGGCATGGGGATGACCTTGGAGTCGTATTTCCATGGCTCCTGACCGAGGACGGA

amdS

3361 TTTGGTGAAGAGGCGGAGGTCTAACATACTTCATCAGTGACTGCCGGTCTCGTATATAGTATAAAAAGCA

amdS

3431 AGAAAGGAGGACAGTGGAGGCCTGGTATAGAGCAGGAAAAGAAGGAAGAGGCGAAGGACTCACCCTCAAC

amdS

3501 AGAGTGCGTAATCGGCCCGACAACGCTGTGCACCGTCTCCTGACCCTCCATGCTGTTCGCCATCTTTGCA

amdS

3571 TACGGCAGCCGCCCATGACTCGGCCTTAGACCGTACAGGAAGTTGAACGCGGCCGGCACTCGAATCGAGC

amdS

3641 CACCGATATCCGTTCCTACACCGATGACGCCACCACGAATCCCAACGATCGCACCCTCACCACCAGAACT

amdS

3711 GCCGCCGCACGACCAGTTCTTGTTGCGTGGGTTGACGGTGCGCCCGATGATGTTGTTGACTGTCTCGCAG

amdS

3781 ACCATCAGGGTCTGCGGGACAGAGGTCTTGACGTAGAAGACGGCACCGGCTTTGCGGAGCATGGTTGTCA

amdS

3851 GAACCGAGTCCCCTTCGTCGTACTTGTTTAGCCATGAGATGTAGCCCATTGATGTTTCGTAGCCCTGGTG 000033

amdS

```
3921    GCATATGTTAGCTGACAAAAAGGGACATCTAACGACTTAGGGGCAACGGTGTACCTTGACTCGAAGCTGG
                                          amdS
3991    TCTTTGAGAGAGATGGGGAGGCCATGGAGTGGACCAACGGGTCTCTTGTGCTTTGCGTAGTATTCATCGA
                                          amdS
4061    GTTCCCTTGCCTGCGCGAGAGCGGCGTCAGGGAAGAACTCGTGGGCGCAGTTTGTCTGCACAGAAGCCAG
                                          amdS
4131    CGTCAGCTTGATAGTCCCATAAGGTGGCGTTGTTACATCTCCCTGAGAGGTAGAGGGGACCCTACTAACT
                                          amdS
4201    GCTGGGCGATTGCTGCCCGTTTACAGAATGCTAGCGTAACTTCCACCGAGGTCAACTCTCCGGCCGCCAG
                                          amdS
4271    CTTGGACACAAGATCTGCAGCGGAGGCCTCTGTGATCTTCAGTTCGGCCTCTGAAAGGATACCCGATTTC
                                          amdS
4341    TTTGGGAAATCAATAACGCTGTCTTCCGCAGGCAGCGTCTGGACTTTCCATTCATCAGGGATGGTTTTTG
                                          amdS
4411    CGAGGCGGGCGCGCTTATCAGCGGCCAGTTCTTCCCAGGATTGAGGCATTCTGTGTTAGCTTATAGTCAG
                                          amdS
4481    GATGTTGGCTCGACGAGTGTAAACTGGGAGTTGGCATGAGGGTTATGTAGGCTTCTTTAGCCCCGCATCC
                                          amdS
4551    CCCTCATTCTCCTCATTGATCCCGGGGGAGCGGATGGTGTTGATAAGAGACTAATTATAGGGTTTAGCTG
                                          amdS
4621    GTGCCTAGCTGGTGATTGGCTGGCTTCGCCGAATTTTACGGGCCAAGGAAAGCTGCAGAACCGCGGCACT
                                          amdS
4691    GGTAAACGGTAATTAAGCTATCAGCCCCATGCTAACGAGTTTAAATTACGTGTATTGCTGATAAACACCA
                                          amdS
4761    ACAGAGCTTTACTGAAAGATGGGAGTCACGGTGTGGCTTCCCCACTGCGATTATTGCACAAGCAGCGAGG
                                          amdS
4831    GCGAACTTGACTGTCGTCGCTGAGCAGCCTGCAGTCAAACATACATATATATCAACCGCGAAGACGTCTG
                                          amdS
4901    GCCTTGTAGAACACGACGCTCCCTAGCAACACCTGCCGTGTCAGCCTCTACGGTTGTTACTTGCATTCAG
                                          amdS
4971    GATGCTCTCCAGCGGGCGAGCTATTCAAAATATTCAAAGCAGGTATCTCGTATTGCCAGGATTCAGCTGA
                                          amdS
                                                                          pUC19
                                                                          ~~~~~~
5041    AGCAACAGGTGCCAAGGAAATCTGCGTCGGTTCTCATCTGGGCTTGCTCGGTCCTGGCGTAGATCTAGAG
                                          amdS

                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5111    TCGACCTGCAGGCATGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5181    CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATT
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5251    AATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5321    CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5391    CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGG
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5461    GGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTG
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
               ColE1 origin
               ~
5531    CTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5601    GAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5671    GACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCA
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5741    CGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5811    AGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCC
                                       pUC19
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5881    ACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAG
```

000034

```
                                        pUC19
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5951    TGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCT
                                        pUC19
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6021    TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTG
                                        pUC19
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6091    CAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGAC
                                        pUC19
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6161    GCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGA
                    pUC19
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                        Pura3
                                                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6231    TCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTATTTTCAATTCAATTCATCATTTTTTTTTTATTC
                                        Pura3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6301    TTTTTTTTGATTTCGGTTTCTTTGAAATTTTTTTGATTCGGTAATCTCCGAACAGAAGGAAGAACGAAGG
                                        Pura3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6371    AAGGAGCACAGACTTAGATTGGTATATATACGCATATGTAGTGTTGAAGAAACATGAAATTGCCCAGTAT
                            Pura3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                        URA 3
                                                                ~~~~~~~~~~~~~~~~~~~~~
6441    TCTTAACCCAACTGCACAGAACAAAAACCTGCAGGAAACGAAGATAAATCATGTCGAAAGCTACATATAA
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6511    GGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAGCTATTTAATATCATGCACGAAAAGCAAACA
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6581    AACTTGTGTGCTTCATTGGATGTTCGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCA
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6651    AAATTTGTTTACTAAAAACACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCT
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6721    AAAGGCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACA
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6791    GTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAATGCACACGGTG
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6861    TGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAGTAACAAAGGAACCTAGAGGCCT
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6931    TTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTATCTACTGGAGAATATACTAAGGGTACTGTTGAC
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
7001    ATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAG
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
7071    GTTACGATTGGTTGATTATGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTA
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
7141    TAGAACCGTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTGCAAAG
                                        URA 3
                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
7211    GGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAGATGCG
                                                Tura3
                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       URA 3
       ~~~~~~~~~~~
7281    GCCAGCAAAACTAAAAAACTGTATTATAAGTAAATGCATGTATACTAAACTCACAAATTAGAGCTTCAAT
                Tura3
       ~~~~~~~~~~~~~~~~~~~~~~~~~
7351    TTAATTATATCAGTTATTACCCATG
```

000035

[redacted]

Appendix 2

000036

Appendix 2 Protein sequences of the HL1232 lipase, the *T. lanuginosus* lipase and the *F. oxysporum* lipase.

Sequence of the HL1232 lipase:

```
  1  MRSSLVLFFV SAWTALASPI RREVSQDLFN QFNLFAQYSA AAYCGKNNDA
 51  PAGTNITCTG NACPEVEKAD ATFLYSFEDS GVGDVTGFLA LDNTNKLIVL
101  SFRGSRSIEN WIANLNFWLK KINDICSGCR GHDGFTSSWR SVADTLRQKV
151  EDAVREHPDY RVVFTGHSLG GALATVAGAD LRGNGYDIDV FSYGAPRVGN
201  RAFAEFLTVQ TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKSGTLV
251  PVTRNDIVKI EGIDATGGNN QPNIPDIPAH LWYFQATDAC NAGGFSWRRY
301  RSAESVDKRA TMTDAELEKK LNSYVQMDKE YVKNNQARS
```

Position 1 to 284 is a part of the mature *T. lanuginosus* lipase (indicated in red). Position 285 to 339 is the C-terminal of the *F. oxysporum* lipase (indicated in blue). Residue 113, 118 and 121 are altered by site directed mutagenesis (G113A, D118W and E121K) (indicated in frame).

Sequence of the *T. lanuginosus* lipase:

```
  1  MRSSLVLFFV SAWTALASPI RREVSQDLFN QFNLFAQYSA AAYCGKNNDA
 51  PAGTNITCTG NACPEVEKAD ATFLYSFEDS GVGDVTGFLA LDNTNKLIVL
101  SFRGSRSIEN WIGNLNFDLK EINDICSGCR GHDGFTSSWR SVADTLRQKV
151  EDAVREHPDY RVVFTGHSLG GALATVAGAD LRGNGYDIDV FSYGAPRVGN
201  RAFAEFLTVQ TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKSGTLV
251  PVTRNDIVKI EGIDATGGNN QPNIPDIPAH LWYFGLIGTC L
```

Sequence of the *F. oxysporum* lipase:

```
  1  MLLLPLLSAI TLAVASPVAL DDYVNSLEER AVGVTTTDFS NFKFYIQHGA
 51  AAYCNSEAAA GSKITCSNNG CPTVQGNGAT IVTSFVGSKT GIGGYVATDS
101  ARKEIVVSFR GSINIRNWLT NLDFGQEDCS LVSGCGVHSG FQRAWNEISS
151  QATAAVASAR KANPSFNVIS TGHSLGGAVA VLAAANLRVG GTPVDIYTYG
201  SPRVGNAQLS AFVSNQAGGE YRVTHADDPV PRLPPLIFGY RHTTPEFWLS
251  GGGGDKVDYT ISDVKVCEGA ANLGCNGGTL GLDIAAHLHY FQATDACNAG
301  GFSWRRYRSA ESVDKRATMT DAELEKKLNS YVQMDKEYVK NNQARS
```

[redacted]
000037

[redacted]

Appendix 3

novozymes

Preliminary Product Sheet

February 10th 2002

Lecitase Ultra

Description

Lecitase Ultra is a protein engineered carboxylic ester hydrolase (E.C.3.1.1.3) from *Thermomyces lanuginosus/Fusarium oxysporum* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* micro-organism.
Lecitase Ultra has inherent activity towards both phospholipid and triglyceride structures. The activity towards triglyceride can be controlled in the applications, when the respective recommendations described below are followed. Lecitase Ultra acts on phospholipids (phosphatides, lecithins) as a phospholipase type A1 to yield the corresponding lyso-1-phospholipid plus free fatty acid (FFA). In practical applications acyl migration will result in conversion to the more stable lyso-2-phospholipds, - same result as achieved by reaction with A2-phospholipase.

Product Properties

Product Type

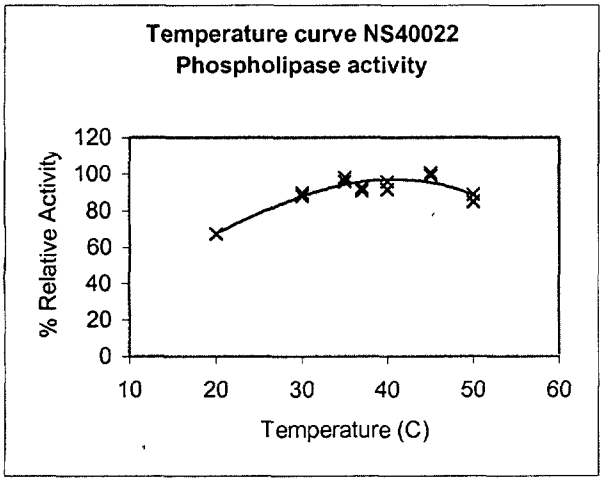
Lecitase Ultra is a pale brownish liquid. The color may vary from batch to batch and color intensity is not an indication of product strength. The density of Lecitase Ultra is approx. 1.2 g/cm3

Activity

Lecitase Ultra is a liquid preparation standardized in Lipase Units per g (LU/g).
One unit is equivalent to the amount of enzyme producing 1 mole of titratable butyric acid per minute under the standard conditions.
1 KLU = 1,000 LU

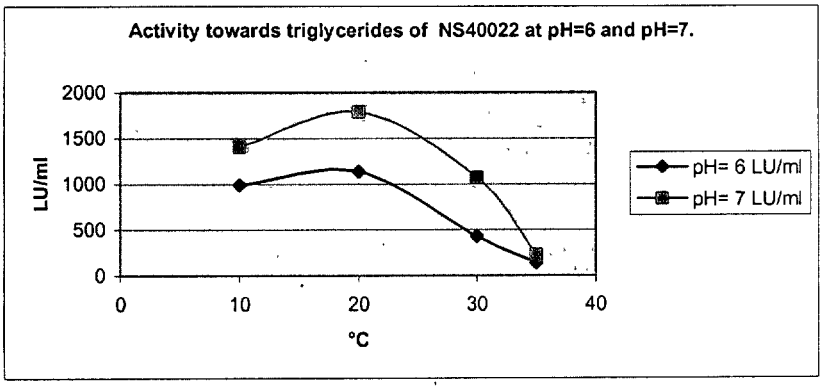
When stored in closed containers at 25° C, (Product name of enzyme) will maintain its declared activity for at least 3 months. Extended storage and/or adverse conditions, including higher temperature or high humidity, may lead to higher dosage requirement."

The activity of Lecitase Ultra (NS40022) is dependent on temperature as illustrated in figure1 below.



000039

The activity towards triglycerides is pronounced at low temperature as seen on figure 2 below.



The conclusion from reading the two figures together is that processing at temperature above 40°C will allow the phospholipase activity to dominate and suppress the lipase activity.

Food-grade status

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Co-dex (FCC).

Applications

Lecitase Ultra is intended for:

- Egg yolk modification
- Hydrolysis of lecithin
- Vegetable oil de-gumming

Reaction Parameters

The optimal working conditions for Lecitase Ultra will vary depending on the actual application and conditions. The following section includes our starting recommendation based on testing to date. As the product is still in an experimental stage, the conditions in the suggested applications may not have been fully optimized.

1. Egg yolk modification

Liquid egg yolk is heated to 50-55°C and added the enzyme in a dosage corresponding to 10-40 KLU/kg egg yolk. Reaction time is 3-4 hours.

Safety

Enzymes are proteins and inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact, The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust. All spills, even small spills should be removed immediately. Use respiratory protection. Major spills should be carefully shoveled into plastic lined containers. Small spills and remains of large spills should be removed by vacuum cleaning or flushing with water (no splashing).

Material Safety Data Sheets are supplied with all products. Further information describing how to handle the product safely is available upon request

000040

Appendix 4

Toxicology

Date: February 2002
Ref.: DSBr
File: 2002-03562-01

SUMMARY OF TOXICITY DATA

Lipase from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae*

Authors:
Ditte Sidelmann Brinch
Peder Bjarne Pedersen

Issued by:
Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

CONTENTS

000043

1. ABSTRACT

Lipase, PPW 7023, is a liquid enzyme concentrate. It is produced by submerged fermentation of a strain of *Aspergillus oryzae*, containing the gene code of a protein-engineered variant of a fungal lipase originating from *Thermomyces lanuginosus*

In the following, toxicity studies carried out with Lipase are summarised.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Huntingdon Life Sciences or at Covance Laboratories, England during the period January 2001 to November 2001.

The main conclusions of the safety studies can be summarised as follows:

Oral administration of Lipase, PPW 7023 to CD rats at dosages of up to 10.0 mL Lipase/kg bw/day (equivalent to 111615 LENU/kg bw/day or 193 KLU/kg bw/day or 1.020 g TOS/kg bw/day) for two weeks was well tolerated and produced no toxicological significant findings.

Oral administration of Lipase, PPW 7023 to CD rats at dosages up to 10.0 mL/kg body weight/day (equivalent to 193466 LU/kg/day, 111615 LENU/kg/day or 1.020 g TOS/kg/day) for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, and this dose was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

Lipase has shown no mutagenic activity in either Ames' test or the chromosome aberration test.

2. TEST SUBSTANCE

2.1 Production of Test Substance

Lipase, PPW 7023, is a liquid enzyme concentrate. The principal enzyme activity is a triacylglycerol acylhydrolase (EC 3.1.1.3), which cleaves the esterbonds in position 1 and 3 of triglycerides. It is a protein-engineered variant of a wild-type lipase from a strain of *Thermomyces lanuginosus*. This enzyme is expressed in a strain of *Aspergillus oryzae* and the product is produced by submerged fermentation and recovered by purification/ concentration of the fermented culture broth.
The Lipase batch, PPW 7023, used for the present toxicological programme, was a mixture of 3 identically produced sub-batches.

2.2 Characterisation

Batch PPW 7023, a dark brown liquid was used for all the studies.

Table 1. Characterisation of Lipase, PPW 7023

	Batch No. PPW 7023
Activity, LENU/g	10500
Activity KLU/g	18.2
Water (KF)	86.9%
Dry matter	13.1%
Ash (600°C)	3.5%
Total Organic Solids (TOS[1])	9.6%
Specific gravity (g/mL)	1.063

[1] %TOS = 100%- % water- % ash- % diluents

2.3 Production Microorganism

Lipase is produced by a strain of *Aspergillus oryzae*. This genetically modified production strain meets the criteria for a safe production microorganism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

A. oryzae is generally regarded as non-pathogenic and non-toxigenic. This species has long history of safe use and it has been used since the beginning of this century in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.
In the present host strain the ability to produce the secondary metabolites cyclopiazonic acid and Kojic acid has been deleted. Further analysis demonstrated the absence of the mycotoxins Aflatoxin B1, Sterigmatosystin, Ochratoxin A, Zearalenone, and T-2 toxin in each of the sub-batches which constitute PPW 7023.

The test substance does not contain this production strain. Absence of the production strain is part of the complete specification of the product.

3. TOXICITY DATA

3.1 Subacute Toxicity

3.1.1 Toxicity Study by Oral Administration to CD Rats for 2 Weeks

A subacute toxicity study in rats with a duration of two weeks served as a dose range finding study to the 13-week study.

Three groups each of five male and five female CD rats received Lipase, PPW 7023 orally by gavage, at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day (equivalent to 11162, 36833 or 111615 LENU/kg bw/day, or 19, 64 or 193 KLU/kg bw/day or 0.102, 0.337 or 1.020 g TOS/kg bw/day) for two weeks. A similarly constituted group received the vehicle (purified water obtained by reverse osmosis) and served as the negative control.

At the end of 2 weeks treatment, all animals were killed and subjected to necropsy.

There were no signs related to treatment and no animals died prematurely. Bodyweight, food consumption and food conversion efficiency were unaffected by treatment with Lipase, PPW 7023. There were no treatment-related haematological and biochemical changes and no treatment-related organ weight or macroscopic changes after two weeks of treatment.

It was concluded that oral administration of Lipase, PPW 7023 to CD rats at dosages of up to 10.0 mL/kg bw/day for two weeks was well tolerated and produced no toxicological significant findings. Consequently, dosages up to 10.0 mL/kg bw/day (equivalent to 111615 LENU/kg bw/day or 193 KLU/kg bw/day or 1.020 g TOS/kg bw/day) are considered appropriate for use in a 13 week toxicity study in this strain of rat.

3.2. Subchronic Toxicity

3.2.1 Toxicity Study by Oral Administration to CD rats for 13 Weeks

The study was carried out in accordance with the OECD guideline 408 (as revised in 1997). It was conducted in accordance with Good Laboratory Practice.
Three groups of 10 male and 10 female CD rats were dosed Lipase, PPW 7023 by gavage at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day. This corresponds to 19347, 63844 or 193466 LU/kg/day, 11162, 36833 or 111615 LENU/kg/day or 0.102, 0.337 or 1.020 g TOS/kg/day. A similar constituted group received the vehicle (purified water obtained by reverse osmosis) and served as the negative control.

Analysis of achieved concentration was performed on samples taken once during weeks 1 and 13. Achieved concentration was evaluated by measurement of enzyme activity, LU/g.

There were no signs related to treatment. Bodyweight gain, food consumption and food conversion efficiency were unaffected by treatment. There were no treatment-related ocular findings. The functional observation battery procedures did not indicate any changes that were attributable to treatment. There were no treatment-related haematological changes. Treatment-related changes in plasma during Week 13 were confined to slightly low cholesterol concentration in males receiving 10.0 ml/kg/day. The changes were not associated with any histopathological changes and were therefore not considered to be of toxicological significance. There were no treatment-related organ weight differences, macroscopic or histopathological changes after 13 weeks of treatment. The results from the content check analysis showed that the measured activity was as expected in week 1 and 13.

It was concluded that the oral administration of Lipase, PPW 7023 to CD rats at dosages up to 10.0 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10.0 mL/kg/day day (equivalent to 193466 LU/kg/day, 111615 LENU/kg/day or 1.020 g TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

3.3. Mutagenic Potential

3.3.1. Bacterial Mutation Assay (Ames Test)

Lipase was examined in a bacterial reverse mutation assay in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) in order to determine the ability to induce gene mutations in bacteria.

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

Lipase (PPW 7023) significantly support growth of the histidine-requiring *S.typhimurium* strains by direct incorporation of the test substance on selective agar media. In order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied. Bacterial cultures were exposed to Lipase, solvent and appropriate positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.
The part of the study comprising the *E.coli* strain was conducted by direct plate incorporation.

Two independent mutation tests were performed in the presence and absence of an activating system derived from rat liver (S9 mix). All bacterial strains were exposed to serial dilutions of Lipase and the final concentrations of the test article achieved were 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg per ml (*S.typhimurium)* or per plate (*E.coli*).
The number of revertants per plate was determined by triplicate plating at each dose on selective agar. The number of viable bacteria in each culture was determined by plate count.
The sensitivity of the individual bacterial strains and the metabolising potential of the S-9 mix were confirmed in both studies by significant increases in number of revertant colonies induced by diagnostic mutagens under similar conditions.

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Lipase, either in the presence or absence of S-9 mix.

It was concluded, that Lipase, PPW 7023, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

3.4.2 Chromosome Aberration Assay

The effects on chromosomal structure of exposure to Lipase were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997).

Heparinized whole blood cultures, pooled from male donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.
Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction

(S-9) from animals induced with Aroclor. Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with Lipase covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/mL. The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Lipase on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Lipase treatments and negative controls. Slides were scored blind and aberrations recorded according to international classifications.

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed. In the first experiment the highest concentration of Lipase chosen for analysis, 5000 µg/mL, produced approximately 3% and 1% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 5000 µg/mL produced approximately 0% and 22% mitotic inhibition in the absence and presence of S-9, respectively.

Cells treated with Lipase, either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. The one exception to this was a marginal increase in only one of duplicate cultures observed at the highest dose level in the 1. experiment in the absence of S-9. This increase was small and neither reproduced between replicate cultures nor in the 2. experiment. Therefore it was considered spurious and of no biological significance. Beyond this no reproducible increases in aberration frequency that were significantly higher than those observed in the negative controls or that fell outside the historical negative control ranges.
Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

Lipase (Batch No. PPW 7023), under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes when tested to a concentration of 5000 µg/mL in either the absence or presence of S-9.

4. CONCLUSION

The results of the toxicological studies outlined above did not reveal any adverse effect of Lipase, PPW 7023. Based on these toxicological data and the fact that the production strain reportedly has a history of safe use, it is our conclusion that there are no reasons for safety concerns when using this Lipase as a food additive. In our opinion Lipase can be considered generally recognized as safe.

5. REFERENCES

5.1 Study Reports

Huntingdon Life Sciences: Study No.: JLY 006. Novozymes Reference No.: 20016008. Lipase, PPW 7023: Toxicity Study by Oral Administration to CD Rats for 2 Weeks. June 2001.

Huntingdon Life Sciences: Study No.: JLY 007. Novozymes Reference No.: 20006009. Lipase, PPW 7023: Toxicity Study by Oral Administration to CD Rats for 13 Weeks. November 2001.

Novozymes A/S Report No.: 2001-17430-01. NN Study No.: 20018059. Lipase (Batch No. PPW 7023): Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli.*

Covance Laboratories Limited Report No.1974/6. Novozymes Study No.20016018 Lipase: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte.

5.2 Guidelines

EEC, 1992. Annex to Commission Directive 92/69/EEC. *The Journal of the European Communities* L383A, 29 December.

OECD, Guidelines for testing of Chemicals. Section 3 and 4: Health effects. Organisation for Economic Co-operation and Development, Paris.

OECD principles of Good Laboratory Practice (GLP) (as revised in 1997), ENV/MC/CHEM(98)17. OECD, Paris.

End Submission